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Biosynthesis of Sinigrin. VI.[1] Incorporation from Homomethionine(2-^{14}C,^{15}N) and Some Labelled Compounds into Sinigrin

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Some labelled compounds were fed to horseradish leaves (*Armoracia lapathifolia* Gilib.) and yellow mustard seeds (*Brassica juncea* Cosson). DL-Homomethionine(2-^{14}C,^{15}N) was directly incorporated into sinigrin in comparison with the other labelled compounds, however DL-allylglycine(2-^{14}C,^{15}N) was incorporated insignificantly and DL-methionine(2-^{14}C), sodium acetate(2-^{14}C) and sodium malonate(2-^{14}C) were incorporated without randomization. The relationships of their efficient precursors were discussed on sinigrin biosynthesis. Chemical degradation of the allyl thiourea derived labelled mustard oil was described.

Recently various biosynthetical experiments have been reported on the mustard oil glucosides in plants to suggest that the aglucone part is derived from the α-amino acid which has an intimate structural correlation with it.

It was confirmed from the experimental evidences that glucotropaeolin was derived from phenylalanine in garden nasturtium (*Tropaeolum majus* L.),[3,4] L-γ-phenylbutyrine was a precursor of gluconasturtiin in watercress (*Nasturtium officinale* R. Br.)[5] and tryptophan was incorporated into glucobrassicin in cabbage (*Brassica oleracea* L.).[6] In our preliminary communication, we showed that homomethionine was a direct precursor of sinigrin in horseradish (*Armoracia lapathifolia* Gilib.).[7] Recently Chisholm and Wetter also reported that homomethionine was incorporated into sinigrin in horseradish.[8] However Kindl found that *p*-coumaric acid was a more efficient precursor of sinalbin than tyrosine, which was an α-amino acid having a similar structure to sinalbin.[9]

The present paper showed that DL-homomethionine(2-^{14}C, ^{15}N) was directly incorporated into sinigrin in comparison with other some labelled compounds administered into horseradish and yellow mustard (*Brassica juncea* Cosson).

Labelled compounds were administered into the plants, and radioactive allyl isothiocyanate derived from sinigrin was obtained. Allyl isothiocyanate was added to an ammonia solution to be converted to allyl thiourea. Allyl thiourea was degraded stepwise by a method described below, and the activities of the various degradation products were determined. Allyl thiourea was oxidized with hydrogen peroxide and barium hydroxide to afford allylamine and formic acid. Oxidation of formic acid with bromine gave carbon dioxide which was isolated as barium carbonate (C-1). A part of allylamine was isolated as the 2,4-dinitrophenyl derivative and the most part was ozonised to give formaldehyde, isolated as the dimedone derivative (C-4), and 2-aminoacetoaldehyde, which was converted to glycine by permanganate

1) Part V: *Chem. Pharm. Bull.* (Tokyo), **16**, 1128 (1968).
2) Location: *9-1, 4-Chome, Anagawa, Chiba-shi, Chiba.*
3) E.W. Underhill, *et al.*, *Can. J. Biochem.*, **40**, 1505 (1962); *Biochem. Biophys. Res. Comm.*, **14**, 425 (1964).
4) M.H. Benn, *Chem. Ind.*, **1962**, 1907.
5) E.W. Underhill, *Can. J. Biochem.*, **43**, 179 (1965).
6) M. Kutáček, Ž. Precházka, and K. Vereš, *Nature*, **194**, 393 (1962).
7) M. Matsuo and M. Yamazaki, *Biochem. Biophys. Res. Comm.*, **25**, 269 (1966).
8) M.D. Chisholm and L.R. Wetter, *Can. J. Biochem.*, **44**, 1625 (1966).
9) H. Kindl, *Monatsh. Chem.*, **95**, 439 (1964).

oxidation. Glycine was partly isolated as the 2,4-dinitrophenyl derivative and mainly oxidized with ninhydrin to yield carbon dioxide, and formaldehyde. Each of them was converted to barium carbonate (C-3) or the dimedone derivative (C-2). The degradation scheme of sinigrin described above was shown in Chart 1.

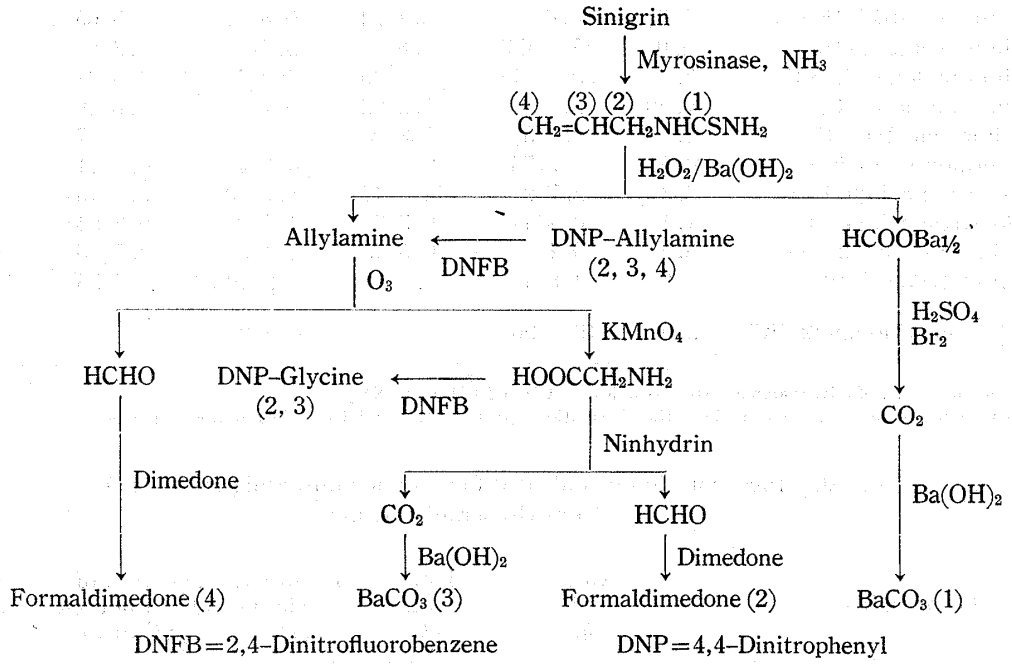

Chart 1. The Degradation Scheme of Sinigrin

The specific radioactivity and the distribution of radioactivity in allyl thiourea obtained from yellow mustard seeds are given in Table I and II, and from horseradish leaves in Table III and IV.

Table I. The Specific Radioactivity of Allyl thiourea obtained from Yellow Mustard Seeds

Compounds[a]	Amt. fed (μCi)	Sp. act. (mCi/mM)	Allyl thiourea	
			Sp. act. (μCi/mM)	Sp. incorporation[b] ($\times 10^{-2}$) %
Sodium acetate(1-^{14}C)	100	6.0	0.07	0.1
Sodium acetate(2-^{14}C)	100	6.0	0.81	1.3
Sodium malonate(2-^{14}C)	100	6.1	0.93	1.5
Sodium formate(^{14}C)	100	4.0	0.11	0.3
Succinic acid(1,4-^{14}C)	100	3.0	0.21	0.7
DL-Methionine($^{14}CH_3$)	100	2.3	0.08	0.3
DL-Aspartic acid(2-^{14}C)	100	1.7	0.23	1.4
DL-Aspartic acid(4-^{14}C)	50	1.5	0.13	0.9
DL-Glutamic acid(2-^{14}C)	100	1.4	0.14	1.0
DL-Allylglycine(2-^{14}C)	50	0.45	0.005	0.1
DL-Homomethionine(2-^{14}C)	50	0.45	0.11	2.4

a) Each 0.03 mM of precursor was administered.

b) Sp. incorporation $= \frac{\text{Sp. act. } (\mu\text{Ci/mM}) \text{ of allyl thiourea}}{\text{Sp. act. } (\mu\text{Ci/mM}) \text{ of administered compound}} \times 100$

Table II. The Distribution of ^{14}C in Allyl thiourea obtained from Yellow Mustard Seeds

Compounds[a]	Allyl thiourea Sp. act. dpm/mM ($\times 10^4$)	Each carbon sp. act. dpm/mM ($\times 10^3$) C–1	C–2	C–3	C–4[b]	Degradation yield (%)
Sodium acetate(1–^{14}C)	1.3	0.6(5)	3.1(24)	0.8(6)	8.5(65)	96
Sodium acetate(2–^{14}C)	3.0	17 (63)	4.8(19)	2.5(9)	2.3(8)	90
Sodium malonate(2–^{14}C)	7.7	50 (67)	15 (20)	5.0(7)	4.5(6)	98
Sodium formate(^{14}C)	0.59	2.2(40)	1.2(22)	0.4(8)	1.6(29)	92
Succinic acid(1,4–^{14}C)	1.7	0.5(3)	5.2(28)	0.4(2)	12 (67)	105
DL–Methionine($^{14}CH_3$)	0.54	3.7(75)	0.4(10)	0.2(4)	0.5(11)	91
DL–Aspartic acid(2–^{14}C)	3.3	2.7(9)	14 (47)	8.9(30)	3.9(13)	92
DL–Aspartic acid(4–^{14}C)	1.1	0.4(4)	0.3(3)	1.1(9)	9.2(84)	100
DL–Glutamic acid(2–^{14}C)	3.2	0.2(5)	0.5(15)	0.1(3)	2.5(76)	101
DL–Allylglycine(2–^{14}C)	1.2	4.8(4)	3.0(15)	1.2(10)	6.6(55)	103
DL–Homomethionine(2–^{14}C)	2.0	19 (96)		0.4(2)		99

a) The numbering of allyl thiourea carbon as follows; $\overset{4}{C}H_2=\overset{3}{C}H\overset{2}{C}H_2N\overset{1}{H}CSNH_2$
b) The figures in the parentheses indicate the distribution percent of radioactivity of degradation products.

Table III. Incorporation of Labelled Compounds into Sinigrin isolated from Horseradish Leaves

Compounds	Amt. fed (mg)	Total act. (μC)	Fresh wt. of plants (g)	Sp. act. of allyl thiourea (nCi/mM)	Specific incorp.[b] (%)
DL–Methionine–2–^{14}C	8.9	9.4	216	713	0.45
DL–Homomethionine–2–^{14}C	10.2	2.7	212	277	0.65
DL–Homomethionine–G–^{3}H	19.1	300	216	5500	0.22
DL–Allylglycine–2–^{14}C	6.8	2.3	200	4.5	0.01
4–Methylthiobutyramide–1–^{14}C	15.6	30	209	7.4	0.003
3–Methylthiopropionamide–1–^{14}C	7.1	10	212	0.3	0.0002

a) Cultivated for 24 hours
b) Specific incorp. $= \frac{\text{Sp. act. } (\mu\text{Ci/mM}) \text{ of allyl thiourea}}{\text{Sp. act. } (\mu\text{Ci/mM}) \text{ of precursor}} \times 100$

Table IV. The Distribution of ^{14}C in Allyl thiourea obtained from Horseradish Leaves

Compounds	Sp. act. of allyl thiourea ($\times 10^4$) (dpm/mM)	Sp. act. of each carbon ($\times 10^4$) (dpm/mM) C–1	C–2	C–3	C–4
DL–Methionine(2–^{14}C)	10.3	0.52(5)	9.2(92)	0.11(1)	0.25(2)
DL–Homomethionine(2–^{14}C)	6.3	6.2(98)		0(0)	

a) The figures in the parentheses indicate the distribution percent of radioactivity of degradation products.

Table I and III present the data showing the incorporation of ^{14}C from various compounds into sinigrin in yellow mustard seeds and horseradish leaves. In the both cases, the incorporation ratio from homomethionine was significantly higher than that from the other compounds, and homomethinonine was incorporated into allyl thiourea without randomization. These results showed that the most efficient precursor of the aglycone moiety of sinigrin should be homomethionine. This was also confirmed by the double tracer experiments, in which the amino group of homomethionine was incorporated into the molecule of sinigrin being

attached with the intact carbon chain. The double tracer experiments showed that when DL-homomethionine(2-^{14}C, ^{15}N) was fed for 3 hours as a precursor, the initial ^{14}C/^{15}N ratio was unchanged in allyl thiourea obtained (Table V). L-Homomethionine was found by Chisholm and Wetter in horseradish containing sinigrin[8] and isolated from cabbage by Sugii, *et al.*[10] This supports the fact that homomethionine is a direct precursor of sinigrin.

Table V. ^{14}C and ^{15}N Double Lablelled Tracer Experiments in Horseradish Leaves

Metabolic period (hour)	Fresh wt. of plants (g)	Precursor: Amt. fed (mg)	Precursor: Total act. (μCi)	Precursor: Specific act. (μCi/mM)	Precursor: Atoms % excess ^{15}N[b]	Precursor: ^{14}C[a]/^{15}N	Allyl thiourea: Specific act. (μCi/mM)	Allyl thiourea: Atoms % excess ^{15}N[b]	Allyl thiourea: ^{14}C[a]/^{15}N
DL-Homomethionine-2-^{14}C,^{15}N									
3	210	32.4	6.7	33.4	32.4	1.0	0.24	0.22	1.1
24	212	32.4	6.7	33.4	32.4	1.0	0.58	0.32	1.8
L-Allylglycine-2-^{14}C,^{15}N									
24	200	64.3	21.4	38.1	51.0	0.75	0.0054	0.11	0.05

a) $^{14}C/^{15}N = \frac{\mu Ci/mM \text{ of } ^{14}C}{\text{Atoms \% excess of } ^{15}N}$

b) The value was corrected for the nitrogen atom derived from the ammonia used in the preparation of allyl thiourea.

On the other hand, allylglycine whose structure was closely related to the aglycone moiety of sinigrin, was incorporated into singrin with very low ratio, and when allylglycine(2-^{14}C, ^{15}N) was fed to the plants, ^{14}C/^{15}N ratio of sinigrin differed remarkably from that of the precursor. Therefore, the formation of the double bond in sinigrin molecule would be caused by the elimination of methanethiol at the final stage of biosynthesis, and it would be plausible to assume that glucoibervirin, a mustard oil glucoside of *Iberis sempervirens* L., is the immediate precursor of sinigrin, since it is formulated by the addition of methanthiol to the double bond of sinigrin (Chart 2). Meanwhile 4-methylthiobutyramide, a possible precursor of sinigrin, was not incorporated into sinigrin.

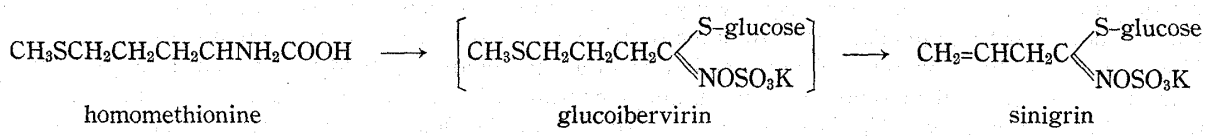

Chart 2. The Biosynthetic Scheme of Sinigrin

Since methionine(2-^{14}C), acetate(2-^{14}C) and malonate(2-^{14}C) were significantly incorporated into sinigrin without randomization, it is suggested that homomethionine may be biosynthesized from methionine and acetate or malonate. We assumed in the preliminary communication that malonate might be more predominant than acetate, as a precursor in sinigrin biosynthesis since the incorporation ratio of sodium acetate(2-^{14}C)was decreased by the addition of sodium malonate. But the results of the time course feeding experiment in young horseradish leaves seems to show that acetate is more effective as a precursor of sinigrin than malonate (Fig. 1). If homomethionine could be derived from a condensation of 3-methylthio-1-oxobutyric acid formed from methionine, with acetate, the biosynthetic pathway seems to be similar with that which leucine is formed by the condensation of acetate with isovalerate, formed from valine, with the subsequent loss of a carboxyl group yielding a

10) M. Sugii, Y. Suketa, and T. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **12**, 1115 (1964).

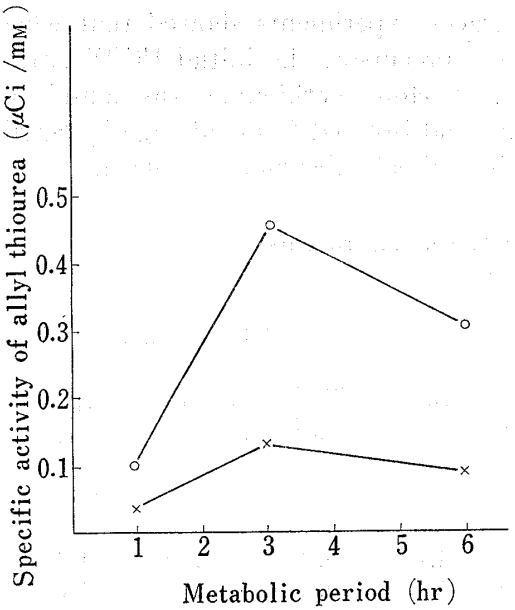

Fig. 1. Incorporation of Sodium Acetate(2-^{14}C)(○—○) and Sodium Malonate(2-^{14}C) (×—×) to Allyl thiourea in Young Horseradish Leaves

keto acid. Chisholm and Wetter proposed that the condensation product of methionine and acetic acid would be α-(2-methylthioethyl)malic acid, which would then be converted to β-(2-methylthioethyl)malic acid.[11]

Methionine is converted to 3-methylthiopropionamide with horseradish leaves and horseradish peroxidase,[12] but the amide was not incorporated into sinigrin and it seems likely that the amide would not be an intermediate of sinigrin and the peroxidase would not relate with sinigrin biosynthesis.

Aspartic acid was incorporated into sinigrin with some extent. Chisholm and Wetter suggested that aspartic acid would give rise to methionine, which might be converted into sinigrin.[11]

Succinic acid, glutamic acid and formic acid are insignificant as the precursors of sinigrin.

Experimental

Labelled Compound and Isotope Analysis——DL-Allylglycine(2-^{14}C) and (^{15}N), DL-homomethionine(2-^{14}C), (G-^{3}H) and (^{15}N), 3-methylthiopropionamide(1-^{14}C) and 4-methylthiobutyramide(1-^{14}C) were synthesized in our laboratory by the methods described in a previous paper.[13] The other labelled compounds fed to the plants were obtained from commercial sources.

The radioactivities were measured using Tri-Carb liquid scintillation spectrometer, series 314X and 314EX (Packard Instrument Company, Inc.) and gas flow counter (Irigaku-Kenkyusho, Inc.). ^{15}N Assay was carried out in the Institute of Physical and Chemical Research by using mass spectrometer.

Administration of Labelled Compounds into Plants and Isolation of Allyl thiourea——Yellow mustard, *Brassica juucea* Cosson, was grown in the field of our Institute. Six-months old plant was transferred to cultivating pot from the field. Labelled compound was administered into the stem of the plant by the cotton wick method after the flowering period. The mature seeds was harvested and ground in an iron mortar with liquid nitrogen. The powdered seeds were added to water and incubated at 37° for 2 hr to give allyl isothiocyanate. After the separation by means of steam distillation, allyl isothiocyanate was converted into allyl thiourea with ammonia. Allyl thiourea solution was evaporated *in vacuo* to remove ammonia. The residue was dissolved in water and extracted with ether to remove impurity. The crude allyl thiourea was treated with a small amount of Norit A and recrystallized from water and EtOH-isopropyl ether. Horseradish, *Armoracia lapathifolia* Gilib., was cultivated in the field of our Institute. The leaves were obtained from 3-months old plants. Labelled compound was administered into leaves by immersing the cut end in the aqeuous solution of tracer. After 24 hr cultivation the leaves was harvested and homogenized in a Waring blender at 0°. The homogenate was incubated at 37° for 2 hr to give allyl isothiocanate, isolated as allyl thiourea.

Degradation of Allyl thiourea——After 30% H_2O_2 (0.85 ml) was added dropwise to allyl thiourea (290 mg) in water (10 ml) at 0° over 20 min under stirring, the stirring was continued at room temperature for 20 min. The reaction mixture was heated for 30 min on a boilling water bath. A solution of $Ba(OH)_2 \cdot 8H_2O$ (1.7 g) in water (40 ml) was added into the cooled mixture. This was refluxed on an oil bath for 30 min. The mixture was distillated to give allylamine. The distillate was led into 10% HCl, and allylamine was isolated as HCl salt. The residual solution was acidified with H_2SO_4 and filtered to remove $BaSO_4$ precipitate. The steam distillation of the filtrate afforded a solution of HCOOH, which was oxidised with Br-water to liberate CO_2 which was isolated as $BaCO_3$ (100 mg). On the other hand, about one tenth of allylamine hydrochloride obtained was converted to 2,4-dinitrobenzene derivative (DNP-allylamine). $NaHCO_3$ (4 g) was added to allylamine hydrochloride in water (5 ml). 2,4-Dinitrofluorobenzene (500 mg) in EtOH (5 ml) was

11) M.D. Chisholm and L.R. Wetter, *Can. J. Biochem.*, **42**, 1033 (1964).
12) M. Mazelis and L.L. Ingraham, *J. Biol. Chem.*, **237**, 109 (1962).
13) M. Matsuo, *Chem. Pharm. Bull.* (Tokyo), **16**, 1030 (1968).

added to the mixture and moreover EtOH (5 ml) was added to it. The reaction mixture was stirred in darkness at room temperature for 2 hr and extracted with ether. The extract was dried over anhyd. Na_2SO_4 and concentrated *in vacuo*. DNP–allylamine was separated from other products by passing a chloroform solution of the residue through a silicic acid column (diam., 3 cm, length, 17 cm). A yellow fraction eluted secondarily with $CHCl_3$ was collected and evaporated *in vacuo*. The residue was recrystallized from H_2O–MeOH. DNP–Allylamine, mp 74°, was obtained. *Anal.* Calcd. for $C_9H_9O_4N_3$: C, 48.43; H, 4.06; N, 18.83. Found: C, 48.63; H, 4.20; N, 18.97. The most part of allylamine hydrochloride dissolved in water (15 ml), was ozonised at 0° for 2 hr. The steam distillation of the reaction mixture afforded formaldehyde, isolated as dimedone derivative. The dimedone derivative was recrystallized from EtOH. The yield was 87 mg. $KMnO_4$ (60 mg) in water (15 ml) containing one drop of conc. H_2SO_4, was added dropwise at 0° under stirring to the residual solution without formaldehyde. After standing overnight the mixture was neutralized with KOH. The solution was filtered to remove MnO_2 and the filtrate contained glycine. About one tenth of glycine was isolated as 2,4–dinitrobenzene derivative (DNP–glycine) with the method described above. DNP–Glycine was purified by passing it through a Celite column prepared from Celite 545 (30 g) and phosphate buffer (20 g, pH 7). Recrystallization from H_2O–MeOH afforded DNP–glycine. mp 202—204° (decomp.). *Anal.* Calcd. for $C_8H_7O_6N_3$: C, 39.84; H, 2.93; N, 17.43. Found: C, 39.57; H, 3.09; N, 17.41. The main part of glycine in water (20 ml) was added to the mixture of ninhydrin (500 mg), K_3PO_4 (350 mg), KH_2PO_4 (2.0 g) and water (10 ml). The mixture was distilled under nitrogen stream. The distillate contained HCHO which was isolated as dimedone derivative (43 mg). The carbon dioxide driven out by the stream of N_2 was led into a saturated $Ba(OH)_2$ solution, and CO_2 was isolated as $BaCO_3$ (90 mg.)

Time Course Feeding Experiment——A solution (5 ml) of sodium acetate(2-^{14}C) (1 mM, 19.3 μCi) or sodium malonate(2-^{14}C)(1 mM, 18.0 μCi) was administered into the one-month old horseradish leaves (100 g). After an appropriate metabolic period, the leaves were soaked into boilling ethanol and sinigrin was isolated from the leaves and converted to allyl thiourea by the method reported previsouly.[14]

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14) M. Matsuo, *Annual Report of National Institute of Radiological Sciences*, **1963**, 28.